Exhibit 8

on Fig. 3*b* represents the area of an Fab-antigen interface[51-53], to indicate that the T-cell receptor, if it binds like an Fab with which it has sequence homology[54], could recognize both the Ia molecule and the peptide simultaneously.

In two other studies of peptide-MHC interaction [OVA (323-339)[32], PCC (93-104)[33]] no simple conformation could be deduced, but the data did not favour an α-helix. These peptides were shown to contain many positions with both MHC and T-cell contact residue characteristics. Such dual character could be expected for some peptide residues, given the structure of the binding site, but it could also arise from reorientation of single substituted peptides within the binding site, thereby obscuring any structural pattern. However, if the actual bound state of these peptides is as conformationally complex as these studies imply, then more detailed structural restrictions on peptide binding are a prerequisite to modelling.

Whereas the above studies locate those peptide residues that contact the MHC molecule, a recent study[22] has implicated I-E^k residue Val114 in contacting the PCC peptide and not the T cell. This is consistent with the model in Fig. 3*a*, since position 114 points up to the site from the β-sheet region and is probably inaccessible to direct interaction with the T-cell receptor[17]. A study showing Lys103 of PCC to contact only the MHC and not the T cell[33], suggests that PCC 103 may contact the β-sheet region of the site.

The hypothetical model for a class II foreign antigen binding site may be useful for formulating testable hypotheses about foreign antigen and T-cell binding. We note, however, that significant shifts in the direction (up to 30°) and location (up to 7 Å) of α-helices and β-strands (20°, 2 Å) have been observed in the X-ray structures of the very closely related members of both the globin and the IgG families[56,68]. Therefore any hypothetical model derived from even closely related sequences may differ from the true structure in important details and should be thought of only as an imperfect guide for experiments.

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Identification of a second human retinoic acid receptor

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We have previously described a human complementary DNA that encodes a novel protein which is homologous to members of the steroid/thyroid nuclear receptor multigene family[1]. This novel protein (*hap* for hepatoma) exhibits strong homology with the human retinoic acid receptor (RAR)[1,2] which has been recently characterized[2,3]. To test the possibility that the *hap* protein might also be a retinoid receptor, a chimaeric receptor was created by replacing the putative DNA binding domain of *hap* with that of the human oestrogen receptor (ER). The resulting *hap*-ER chimaera was then tested for its ability to trans-activate an oestrogen-responsive reporter gene (vit-tk-CAT) in the presence of possible receptor ligands. Here we show that retinoic acid (RA) at physiological concentrations is effective in inducing the expression of this reporter gene by the *hap*-ER chimaeric receptor. This demonstrates the existence of two human retinoic acid receptors designated RAR-α and RAR-β.

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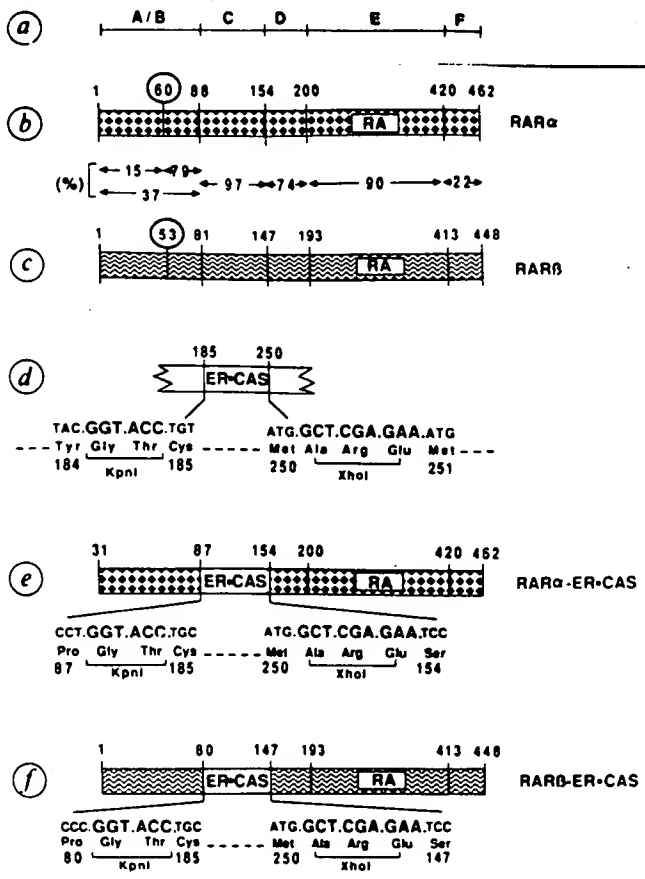

Fig. 1 Schematic representation of the homology between RAR-α and RAR-β and the structures of RAR-ER chimaeric receptors. In agreement with the report of Giguère *et al.*[3], RAR-α (*b*) is represented here as a 462 amino-acid long protein (that is, 30 amino acids longer at the N-terminus than in our initial report[2]. We have found (unpublished results) that the sequences of the RAR-α cDNA clones in our previous report[2] were not colinear with the corresponding genomic sequence upstream of our initiating AUG; in contrast perfect colinearity exists between the 5′ terminal region of the cDNA sequence of Giguère *et al.*[3] and our genomic sequence, substantiating their characterization of the open reading frame (ORF) of RAR-α cDNA). The receptors RAR-α (*b*) and RAR-β (*c*) are divided into six regions, A-F (*a*), by analogy with oestrogen receptors (see text). Numbers denote amino-acid positions. The circled numbers mark the positions of one exon junction, determined from genomic DNA sequence for RAR-α (ref. 2 and unpublished data of the Strasbourg laboratory) and RAR-β[10]. Region E comprises the putative RA binding domain for each receptor. The degree of homology between the receptors is shown between (*b*) and (*c*) (% amino-acid identity). *d* The oestrogen receptor DNA-binding cassette (ER.CAS) of the hER construct HE28[6] comprises the hER C-region (residues 185–205), flanked by unique restriction enzyme sites for *Kpn*I (5′) and *Xho*I (3′). The corresponding sites were engineered on either side of region C of both a truncated form of RAR-α[2] and RAR-β, allowing replacement of those regions by ER.CAS, creating the chimaeric receptors RAR-α-ER.CAS[2] (*e*) and RAR-β-ER.CAS (*f*).
Methods. The construction of RAR-α-ER.CAS (*e*) has been described[2]. RAR-β-ER.CAS was assembled as follows. A 1.4 kilobase DNA fragment containing the entire RAR-β ORF was isolated from a partial digest of the clone λ13 (ref. 1) with *Mae*I; the protruding ends were filled in with Klenow polymerase and the fragment was ligated initially into the *Sma*I site of pTZ18U (United States Biochemicals), yielding the plasmid pCOD20[1]. For mutagenesis and expression studies, the insert was excized from pCOD20 by total digestion with *Bam*HI and partial digestion with *Eco*RI, and re-inserted into the *Eco*RI and *Bam*HI sites of the expression vector pSG5[11] yielding RAR-β0, which can be used to express RAR-β *in vivo* and *in vitro*. Using oligonucleotide-directed mutagenesis as described for RAR-α[2], *Kpn*I and *Xho*I sites flanking region C (codons 81–146) were created in RAR-β0 whereas the *Xho*I site present in the A/B region was removed by mutation. RAR-β region C was then excized and replaced by the ER.CAS, giving the chimaeric receptor RAR-β-ER.CAS (*f*).

Figure 1, *a*–*c* shows a schematic comparison of the cDNA-deduced amino-acid sequences of RAR[2,3] and *hap*[1] proteins (hereafter referred to as RAR-α and RAR-β, respectively). Note that RAR-α is represented as the full-length protein of 462 amino-acid residues[3], instead of the form that we described previously, which lacks 30 amino acids at the N-terminus (ref. 2, see also legend to Fig. 1). RAR-α and RAR-β can be divided into five regions analogous to the A/B, C, D, E and F regions of other members of the nuclear receptor family (refs 4 and 5, Fig. 1*a*). The highest degree of homology (97% amino-acid identity) is found within the 66 residue region C, which in the case of the human glucocorticoid (hGR) and oestrogen (hER) receptors was identified as the DNA-binding domain responsible for the specific recognition of the cognate hormone-responsive elements (refs 6–9 and refs therein). The next most highly conserved region, E (90% amino-acid identity), is a stretch of 220 amino acids which is homologous to the ligand binding domain of the steroid hormone receptors and appears to contain the RA binding domain of RAR-α (ref. 2 and refs therein). Regions C and E are linked by a 46-residue hydrophilic region, D, which is 74% homologous between RAR-α and RAR-β. In contrast, the carboxy-terminal (F) and amino-terminal (A/B) portions of the receptors are much less similar (~22% and 37% amino-acid identity, respectively) and differ in length. A closer comparison of the A/B regions shows that residues 60–87 of RAR-α and 53–80 of RAR-β are 79% identical, whereas there is no significant homology within the remainder of the A/B region. Note in this respect that genomic DNA sequence analyses have located exon boundaries between residues 59/60 of RAR-α[2] and residues 52/53 of RAR-β[10].

These structural homologies suggested to us that, like RAR-α, RAR-β might be a retinoid-inducible transcription factor. To test this hypothesis, we constructed a chimaeric receptor between RAR-β and the human oestrogen receptor, RAR-β-ER.CAS (Fig. 1*f*) in a similar experiment to that used to demonstrate that RAR-α encodes a receptor for RA[2] (see legend to Fig. 1 and ref. 11). To test the efficacy of various ligands to activate the chimaeric receptor, HeLa cells were co-transfected with RAR-β-ER.CAS and a reporter gene containing the oestrogen-responsive upstream sequence of *Xenopus* vitellogenin A2 gene ERE (*vit*) linked to the herpes simplex virus thymidine kinase (*tk*) promoter and the *Escherichia coli* chloramphenicol acetyltransferase gene (CAT) (*vit*-*tk*-CAT[6]). Addition to the culture medium of 10^{-7}M of either thyroid hormone (T3 or T4), vitamin D3 [1,25(OH)$_2$D3], testosterone or oestradiol did not result in any stimulation of *vit*-*tk*-CAT expression by RAR-β-ER.CAS (data not shown). In contrast, a strong stimulation of CAT activity was observed in the presence of 10^{-7}M RA, whereas a much weaker stimulation was achieved with the same concentration of retinol (Fig. 2 and data not shown). The extent of stimulation increased with increasing amounts of transfected RAR-β-ER.CAS with a maximum stimulation of at least 50-fold (Fig. 2*a* and *c*, left panel). This stimulation was similar to that obtained in transfection experiments where the previously described RAR-α chimaeric receptor, RAR-α-ER.CAS, was used to stimulate *vit*-*tk*-CAT expression[2]. No increase in CAT activity was observed when the RAR-β expression vector RAR-β0 was co-transfected with the *vit*-*tk*-CAT reporter gene instead of RAR-β-ER.CAS (data not shown).

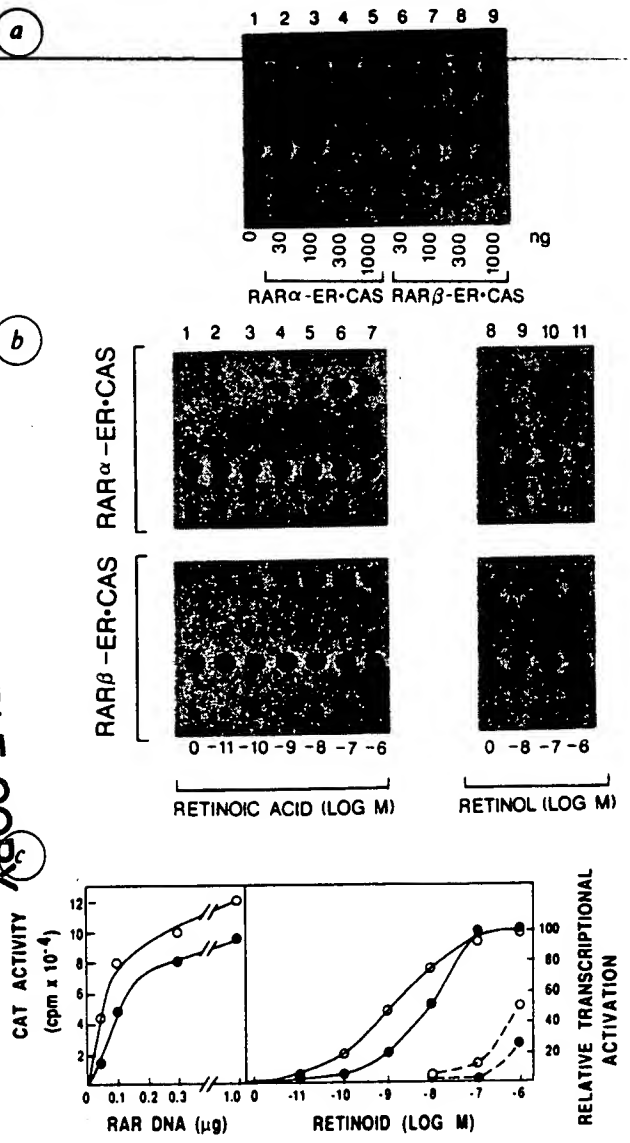

Fig. 2 *a*, CAT activity resulting from activation of the reporter gene *vit-tk*-CAT by the chimaeric receptors RAR-α-ER.CAS and RAR-β-ER.CAS in the presence of RA. From 0–1,000 ng of RAR-α-ER.CAS or RAR-β-ER.CAS, together with 2 μg of *vit-tk*-CAT, were transfected into HeLa cells which were subsequently treated with 10^{-7}M RA. *b*, Effect of RA concentration on the induction of CAT activity by either RAR-α-ER.CAS or RAR-β-ER.CAS. HeLa cells were transfected with 30 ng of either RAR-α-ER.CAS or RAR-β-ER.CAS along with 2 μg of both *vit-tk*-CAT and β-galactosidase control plasmid pCH110 (see below) and then treated with the indicated concentrations of RA or retinol. *c*, **Left panel:** trans-activation of *vit-tk*-CAT by increasing concentrations of RAR-α-ER.CAS (●) or RAR-β-ER.CAS (○). Experiments were similar to those described under (*a*), except that the acetylated forms of ^{14}C-chloramphenicol were isolated from the thin-layer chromatography plates and their radioactivities determined by scintillation counting. **Right panel:** Graph of the results shown in (*b*) for RAR-α-ER.CAS (●) and RAR-β-ER.CAS (○) in response to RA (—) or retinol (- -). Experiments were performed as in (*b*), except that the acetylated forms of ^{14}C-chloramphenicol were isolated, quantified by scintillation counting and the results expressed in per cent of maximal activation. The results displayed in both left and right panels are representative of several independent transfection experiments which gave identical results within 10% variation.

Methods. Transfection experiments were as described[2]. In (*a*) 2 μg of *vit-tk*-CAT reporter DNA, 2 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) and the indicated amounts of the RAR-α or RAR-β chimaeric DNA (plus 16 μg of carrier plasmid DNA) were transfected into HeLa cells. In (*b*) transfections were as in (*a*), but with 30 ng of RAR-α- or RAR-β-ER.CAS. Aliquots of extracts prepared from the transfected cells and corresponding to 1 OD unit of β-galactosidase activity were assayed for CAT activity as previously described[2].

To demonstrate directly that RAR-β binds RA, cytoplasmic extracts prepared from COS-1 cells transfected with the RAR-β0 expression vector were incubated with labelled RA in the presence or absence of excess unlabelled RA or retinol, as described for RAR-α[2]. An increase in the specific, high affinity binding of RA was observed in the transfected cell extracts. As for RAR-α[2], however, there was high background binding in extracts of untransfected cells due to endogenous cellular retinoic acid binding protein (CRABP), thus precluding any accurate determination of the affinity of RA for its receptors (data not shown). The relative affinity of RA for RAR-α and RAR-β was therefore estimated by measuring the activation of *vit-tk*-CAT expression as a function of the ligand concentration under conditions where the reporter gene was present in large excess over the chimaeric receptor (30 ng of RAR-α-ER.CAS or RAR-β-ER.CAS per transfection, see Fig. 2*a* and *c*, left panel). Under these conditions, the concentration of ligand leading to 50% of the maximum inducible CAT activity (ED_{50}) should reflect the relative affinity of the two chimaeric receptors for RA. In agreement with our previous report[2], the ED_{50} for the RAR-α chimaera was close to 10^{-8}M (Fig. 2*b* and *c*, right panel). In contrast, efficiency of RA in stimulating CAT activity was consistently ~10-fold greater with the RAR-β chimaera (ED_{50} ~10^{-9}M), suggesting that RAR-β has a 10-fold greater affinity for RA than RAR-α does. Note that for both RAR-α and RAR-β chimaeras, retinol was ~1,000-fold less efficient than RA at stimulating expression of the reporter gene. As previously discussed for RAR-α, however, we cannot conclude that retinol is directly able to induce trans-activation by RAR-β, as it is known that retinol can be converted to RA in cultured cells[12].

The present data, together with our previous studies[1,2] clearly establish the existence of two structurally closely-related human retinoic acid receptors encoded in two distinct genes which map to different chromosomes. The gene encoding the previously characterized retinoic acid receptor[2,3], designated here as RAR-α, maps to chromosome 17q21.1 (refs 2, 13), whereas the receptor RAR-β, formerly called *hap*, is encoded in a gene that maps to chromosome 3p24 (Mattei, M. G., H. d.T., A.M., P.T. and A.D., manuscript submitted). It is interesting that RAR-α and RAR-β are more homologous to the two closely-related thyroid hormone receptors TRα and TRβ, located on chromosomes 17q11.2 (refs 2, 13) and 3p21–25 (refs 14, 15) respectively, than to any other members of the nuclear receptor family (refs 1–3). These observations suggest that the thyroid hormone and retinoic acid receptors have evolved by gene, and possibly chromosome, duplications from a common ancestor which itself diverged rather early in evolution from the common ancestor of the steroid receptor group of the family. In this respect, we note that the counterparts of the human RAR-α and RAR-

genes are present in both mouse and chicken genomes (unpublished results).

In view of the multiple effects of retinoids on both animal development and homeostasis[2,3,16,17], the identification of a second retinoic acid receptor raises a number of interesting questions. In what tissues are these receptors expressed, to what levels and at what point in development? Preliminary Northern blot analyses of mouse and chicken RNAs from various tissues indicate some specificity in their distribution (unpublished results). Obviously, important clues to the mechanisms through which retinoids control many developmental and homeostatic processes will be obtained by determining the spatial and temporal patterns of expression of the various elements of the retinoid signal transduction system, including both the α and β receptors and the cellular retinoic acid and retinol binding proteins (CRABP and CRBP[2,3,17]). Do RAR-α and RAR-β trans-activate the transcription of different sets of target genes? The high degree of homology between their putative DNA-binding domains (region C, 97% amino-acid identity) suggests that the two receptors might recognize a common RA-responsive element. Their difference in the A/B region, however, may result in differential gene activation, as the corresponding region of the human oestrogen receptor appears to play a specific role in the activation of different oestrogen-responsive genes[7]. Unfortunately, no promoter regions of genes transcriptionally controlled by RA are presently available to test these possibilities.

Our results indicate that RAR-β may mediate activation of transcription by RA at concentrations 10-fold lower than those necessary for activation by RAR-α, although both receptors respond to RA concentrations within the range observed for RA action *in vivo*. As the ED_{50} values for the various biological effects of RA in cell culture span a wide range of concentrations (4×10^{-10} to $>10^{-8}$M (ref. 18)), it is possible that the two RA receptors may be differentially involved in these effects. A concentration gradient of RA across the anterior-posterior axis of the developing chick-embryo limb-bud has recently been described[19], implicating RA as the morphogen in this system. The existence of two receptors for RA, possibly differing in their affinities for the ligand, might play a role in the interpretation of morphogenetic gradients.

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GAL4 activates transcription in *Drosophila*

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GAL4 is a yeast regulatory protein that binds to specific sites within a DNA sequence called UAS_G (galactose upstream activating sequence) and activates transcription of linked genes[1-6]. This activation requires two functions of the protein[7,8]: a DNA binding domain located near the amino terminus[8], and one or more 'activating regions'[7-11]. The 'activating regions' are highly acidic[9-11] (see also ref. 12) and can be replaced, for example, by a short peptide designed to form a negatively charged, amphipathic α-helix[13]. GAL4, as well as deletion derivatives bearing one or more 'activating regions' attached to the DNA binding domain, activates transcription in cultured mammalian cells from mammalian promoters linked to a UAS_G (refs 14, 15). Here we show that GAL4, when expressed in particular tissues of *Drosophila* larvae, stimulates tissue-specific transcription of a *Drosophila* promoter linked to GAL4 binding sites.

We constructed an effector gene that expresses GAL4 in a tissue specific manner in *Drosophila*, and a reporter gene that allows the visualization of GAL4-activated transcription by a histochemical stain for β-galactosidase activity (Fig. 1). In the effector gene, expression of GAL4 is driven by a *Drosophila Adh* promoter. This promoter, when fused to the *lacz* gene of *Escherichia coli*, expresses β-galactosidase mainly in four larval tissues—fat body (fb), Malpighian tubules (mt), anterior midgut (amg) and middle midgut (mmg): expression in the hindgut (hg) and tracheae (tr) is variable (Figs 3, 4; refs 16, 34). In the reporter gene, the TATA-box and 5'-untranslated region of the *Drosophila* heat shock gene *hsp70* are fused to *lacz* (Fig. 1). This fusion gene is transcriptionally inactive (see below): the *hsp70* promoter is normally heat inducible, but sequences upstream of −43 from the *hsp70* transcription start site were deleted, thus eliminating the regulatory elements required for that induction[17-20]. We inserted four copies of a 17 base pair (bp) sequence called the 17-mer upstream of the *hsp70* TATA-box: these 17-mers are closely related to the GAL4 binding sites in UAS_G (ref. 8) and are recognized by GAL4 (ref. 15) (see legend to Fig. 1).

The effector and reporter genes were separately introduced into the *D. melanogaster* genome by P element transformation[21,22] (Fig. 1 legend). Three independent effector transformant lines were separately mated to nine independent reporter transformant lines. Twelve larval progeny of each cross were tested for expression of the reporter gene by a histochemical staining assay for β-galactosidase activity (Fig. 4 legend). The flies transformed with the effector and reporter genes were each heterozygous for the respective P elements (Fig. 1 legend), and so we expected one fourth of their larval progeny to carry one copy of each gene.

In each of the 27 crosses, we detected β-galactosidase activity in ~one quarter of the larval progeny. In all of these larvae, enzyme activity was observed at high levels in the fat body and anterior midgut (Fig. 2), two of the tissues in which we expect the *Adh* promoter of the effector gene to be active. Also as expected, we detected variable levels of β-galactosidase activity in the hindgut and tracheae (Figs 2 and 3). In the middle midgut

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